Universität Zürich[UZH]

Master Thesis

The evolution of an exaggerated male trait and wing morphology in two closely related Sepsid fly species

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Abstract

The relative importance of sexual selection and reproductive character displacement for morphological divergence during speciation is usually difficult to unravel. The black scavenger flies *Sepsis neocynipsea* and *S. cynipsea* provide a good opportunity to address this issue, as they show a geographic distribution with discrete allopatric and sympatric populations. The two species are differentiated on molecular level, but still able to hybridize with less fertile offspring resulting. Eight North American and three European populations of *S. neocynipsea* were compared to seven populations of *S. cynipsea*, which has a range restricted to Eurasia, applying landmark based morphometrics. Geographic patterns of quantitative genetic differentiation of an exaggerated male trait (the armored femur of the foreleg) were compared with patterns of differentiation in geometric wing morphology, using a common garden environment with two temperature regimes in order to estimate trait plasticity. In an additional laboratory study, a replicated mating experiment, with four representative populations of each of the three lineages (*S. neocynipsea* US and EU and *S. cynipsea*), was performed to examine the role of ongoing sexual selection acting on shape and size of the study traits.

In general, stronger divergence in male femur than wing morphology was found, among species and populations. Wing shape differentiation largely followed the phylogenetic expectations, while the phylogenetic signal in male femur shape was much weaker and lineages were mostly separated according to their continent of origin. Since *S. neocynipsea* populations from geographic areas of sympatry with *S. cynipsea* did not diverge faster than allopatric populations, the phylogeographic analysis indicating continuous and ongoing sexual selection to be most important. Strong sexual selection acting on femur shape, but less intense also on wing shape, is supported by the laboratory mating experiment, finding uniform directional selection, favoring males with broad exaggerated femurs, in all study lineages. Male femur shape showed a strong allometric relationship, as it is often the case for exaggerated male secondary traits. A significant latitudinal cline, found for femur size in the American *S. neocynipsea* lineage, also indicate influence of natural selection along an ecological gradient on male secondary trait morphology.

In conclusion the present study documents an interplay of sexual selection and natural selection in shaping male secondary sexual traits, which, however, does not exclude any role of reproductive character displacement. Indeed, both, phylogeographic analysis and laboratory selection experiments, are consistently indicating character displacement as a potential force acting on shape aspects of male for leg morphology.

Zusammenfassung

Die relative Bedeutung von sexueller Selektion und Merkmalverdrängung während der Artbildung ist nur schwer abzuschätzen. Die Schwingfliegen Spezies *Sepsis neocynipsea* und *S. cynipsea* bieten eine gute Gelegenheit, solchen Fragen auf den Grund zu gehen, weil sie eine geografische Verteilung mit klar getrennten allopatrischen und sympatrischen Populationen aufweisen. Trotz molekularer Differenzierung sind zwischenartliche Paarungen möglich, allerdings weist der resultierende Nachwuchs reduzierte Fruchtbarkeit auf. Acht nordamerikanische und drei europäische *S. neocynipsea* Populationen wurden, unter Anwendung eines multivariaten morphometrischen Verfahrens, mit sieben Populationen der nur in Eurasien auftretenden Art *S. cynipsea* verglichen. Die quantitative genetische Differenzierung des stark ausgebildeten und mit Dornen besetzten Femurs männlicher Fiegen wurde mit derjenigen der Flügel verglichen. Unter kontrollierten Laborbedingungen wurden die Populationen beider Arten unter zwei Entwicklungstemperaturen aufgezogen, um die phänotypische Plastizität abzuschätzen und um die Variation auf die genetische Komponente zu reduzieren. In einem weiteren Laborexperiment wurden mit jeweils vier, die drei Linien (*S. neocynipsea* aus Amerika und Europa sowie *S.cynipsea* aus Europa) repräsentierenden Populationen Paarungsexperimente durchgeführt. Diese sollten Aufschluss geben über die Wichtigkeit sexueller Selektion für die Evolution der Form und Grösse der untersuchten Merkmale.

Die Experimente zeigten, dass sich Populationen und Linien grundsätzlich stärker in der Bein- als in der Flügelmorphologie unterscheiden. Die Differenzierung der Flügel entspricht weitgehend den phylogenetischen Erwartungen. Das Differenzierungsmuster der Beine allerdings wiederspiegelt die Phylogenie nur schwach und die Trennung der Linien verläuft hauptsächlich zwischen den Kontinenten. Die Tatsache, dass die europäischen, mit *S. cynipsea* in Sympatrie vorkommenden, *S. neocynipsea* Populationen nicht stärker differenziert sind als die amerikanischen, führt zu dem Schluss, dass fortlaufende sexuelle Selektion der für die Divergenz verantwortliche Faktor ist. Dieser Befund wird unterstützt durch die starke sexuelle Selektion auf Beine und die schwächere sexuelle Selektion, welche im Paarungsexperiment für die Flügel beobachtet wurde. Eine starke Abhängigkeit der Beinmorphologie von der Beingrösse wurde festgestellt. Dies ist oft der Fall in extravagant ausgeprägten männlichen Merkmalen. Eine signifikante Nord-Süd Kline weist zudem auf den Einfluss natürlicher Selektion entlang eines Umweltgradienten hin, welche die Beinmorphologie beeinflusst. Zusammenfassend lässt sich sagen, dass sowohl sexuelle als auch natürliche Selektion zur Evolution der männlichen Vorderbeinmorphologie beitragen. Zusätzlich

deuten sowohl der vergleichende populationsgenetische als auch der experimentelle Ansatz auf potentiellen Einfluss von Merkmalverdrängung hin.

Introduction

Speciation and geographical variation

In order to comprehend the immense species richness in the insect clade and animals in general, the driving processes leading to the splitting of lineages need to be understood. Speciation relies on the emergence of reproductive barriers, which can be induced by a variety of different factors. One, and certainly the most obvious, way how speciation can proceed is through geographic barriers, leading to separated gene pools across a species range. Due to geographic isolation, genetic drift and natural selection have their way evened out, and given enough time can lead to population differentiation and eventually speciation (Lande, 1980). Evidence for allopatric speciation is abundant and has been demonstrated for various taxa, including birds (Coyne & Price, 2000), ticks (Beati et al., 2013) and amphipods (Stevens & Hogg, 2004). Speciation in sympatry and parapatry are theoretically more intriguing because of the homogenizing effect of gene flow (Slatkin, 1985, 1987). With gene flow reduced to a contact zone (parapatry), selective forces and drift determine the speed of divergence and can outbalance gene flow even on quite small scales (Turelli et al., 2001). As theory and empirical examples have shown, the homogenization through gene flow can also be outperformed in sympatry. In heterogeneous environments, disruptive selection favoring ecologically specialized phenotypes may occur and promote genetic divergence within species (Grant, 1999; Barluenga et al., 2006). Assortative mating (Bush, 1966, 1969; Baylis, 1976) and reduced fitness of intermediate phenotypes (Svedin et al., 2008) may then complete the process of speciation.

Sexual selection and character displacement

Sexual selection on mate and gamete recognition traits is considered a potent force, facilitating the evolution of reproductive isolation and speciation (Albert et al., 2000; Puniamoorthy et al., 2010; Soto et al., 2013). Due to high variance in male mating and/or fertilization success, sexual selection can be much stronger than natural selection and lead to rapid diversification of reproduction related traits even beyond their natural fitness optima (Hosken & House, 2011). Sexual selection can act prior, during or after copulation and comprise different mechanisms, namely female choice (Majerus, 1986; Blanckenhorn et al., 2000), male-male competition (Le Boeuf, 1974; Arak, 1983; Moller & Ninni, 1998; Able, 1999) and sexual conflict (Gavrilets, 2000). Numerous behavioural

(e.g. Albert et al., 2000), physiological (Eberhard & Cordero, 1995) and physical traits, such as body size (Wilbur et al., 1978; Price, 1984; Puniamoorthy et al., 2012; Rohner et al., 2016), the size and shape of reproductive ornaments (David et al., 1998) or coloration (Endler, 1980; Albert et al., 2000) are reported to evolve extremely fast in response to sexual selection. In insects, for example, genital structures are cited to diverge much faster than other morphological traits, most likely due to intense sexual selection (Arnqvist, 1998; Hosken & Stockley, 2004; Puniamoorthy et al., 2008; Eberhard, 2013).

In contrast to sexual competition and conflict, which can act more or less continuously within a given population, reproductive character displacement predicts rapid trait diversification only during early stages of speciation in geographic areas of coexistence (Brown Jr. & Wilson, 1956; Sætre et al., 1997; Geyer & Palumbi, 2003). If closely related species are still able to hybridize, the resulting offspring often show reduced fitness. Low hybrid fitness can be due to less attractive (Jiggins et al. 2001; Naisbit et al. 2001; Latour et al., 2014), viable (Hatfield & Schluter, 1999; Price, 2006) or fertile offspring (Svedin et al., 2008). Also, intermediate phenotypes can show dysfunctional behaviors (Pashley & Martin, 1987; Davies et al., 1997; Bel & Gerhardt, 2003). In all of these cases, individuals with better species-discrimination ability are favored by selection and consequently the evolution of reproductive isolation can be reinforced. However, empirical studies that unambiguously distinguish reproductive character displacement from sexual selection at earlier stages of the speciation continuum (Wu, 2001) are scarce because they often lack a control for comparison (Servedio & Noor, 2003). For example, most studies comparing populations from the allopatric peripheries of the species ranges with central populations where the species co-occur tend to neglect the influence of gene flow within the species range (Lofthus-Hills & Littlejohn, 1992; Schilthuizen & Lombaerts, 1995; Holyńska, 2000;). By consequence, the involvement of reinforcement causing population differentiation and speciation may be more widespread than often thought, but undetectable because the signature gets erased by gene flow (Servedio & Noor, 2003).

The genus *Sepsis*

The genus *Sepsis*, within the family of the Sepsid flies (Diptera:Sepsidae), provides an attractive system to investigate the evolutionary forces leading to morphological diversification during different stages of speciation. With about 320 species described, Sepsids are a relatively small family of dung flies with a well resolved phylogeny (Zhao et al., 2013). Many species evolved striking diversity in foreleg morphology, which is frequently used to delineate closely related, otherwise morphologically indistinguishable, species and which may have evolved in response to

sexual selection (Eberhard, 2002b; Blanckenhorn et al., 2004; Ang et al., 2008; Puniamoorthy et al., 2008, 2009; Dmitriew & Blanckenhorn, 2012). The premating behavior is varying in type and intensity throughout the phylogeny, from female choice and resistance (Blanckenhorn et al., 2000; Puniamoorthy et al., 2012) to male-male competition (Ward, 1983; Ward et al., 1992).
Despite extensive research on sexual selection, no studies have tried to assess the relative importance of different evolutionary forces for speciation in this genus. The questions whether sexual selection on morphology acts continuously or only during the immediate speciation process and how it is interplaying with other evolutionary forces, like reinforcement, as yet remain largely unresolved.

The sister species *S. cynipsea* and *S. neocynipsea* are still able to hybridize, but intermediate genotypes have reduced fertility (Giesen et al., unpublished data). Furthermore, whilst *S. neocynipsea* is existent as a North American and a European lineage, *S. cynipsea* has its range restricted to Eurasia (Ozerov, 2005). The factual exclusion of gene flow between the allopatric ranges of *S. neocynipsea* makes it an outstanding system to detect signatures of character displacement as well as concurrent sexual selection, presumably leading to morphological divergence between species and populations. In this study, a common garden environment, including two temperature regimes, is used to compare geographic patterns of quantitative genetic lineage and population differentiation of the armored foreleg femur with patterns of differentiation in wing morphology. Sexual selection is assumed to only play a minor role in the evolution of wing morphology. In addition, I performed a mating experiment, using several populations of all three lineages, in order to test for ongoing sexual selection affecting the size and shape of these two traits. My results provide new insights into the evolutionary dynamics and processes contributing to morphological divergence of a secondary sexual trait at different stages of speciation.

Methods and Materials

Geographic sampling and common garden rearing

For the comparative lineage analysis of male foreleg and wing morphology, 228 iso-female lines of *S. cynipsea* and *S. neocynipsea* were used. *S. cynipsea* originated from seven distinct locations in Central and Northern Europe. North American *S. neocynipsea* originated from eight different populations collected in the United States of America and Canada. For comparison, three European populations of *S. neocynipsea* from the Swiss Alps, where they co-occur with *S. cynipsea*, were examined (Map: *Figure 1*, detailed table of locations and sample sizes see *Appendix table 1*). The flies were raised in a common garden environment under two temperature regimes (18º Celsius and 24º C) at controlled density and unlimited food conditions (sugar, water and cow dung) for one generation. After emergence, the flies were stored in 70% EtOH at -18º C and, for each line and temperature regime, three randomly chosen males were used for morphometric analysis.

Sexual selection experiment sampling

In order to address the role of sexual selection acting on male foreleg and wing morphology, four populations of each of the three phylogenetic lineages were used (Map: *Figure 1*, detailed table of locations and sample sizes see *Appendix table 2*). For each population, four experimental replicates were conducted similar to previous comparative sexual selection experiments on body size across New and Old world populations of *S. punctum* (Puniamoorthy et al., 2012) and *S. neocynipsea* (Rohner et al., 2016). An operational sex ratio of two was chosen for the mating trials, meaning that five females were allowed to mate with 10 males within a period of two hours. Experiments took place in transparent 1l plastic containers equipped with dung, sugar and water, as described in Rohner et al. (2016). In total 40 out of 48 mating trials resulted in at least one successful copulation. This yielded a total 109 males that copulated and 267 males without mating success. During copulation pairs were removed from the observation containers and stored in 70% EtOH, while unsuccessful males were stored separately at the end of the two hours observation period.

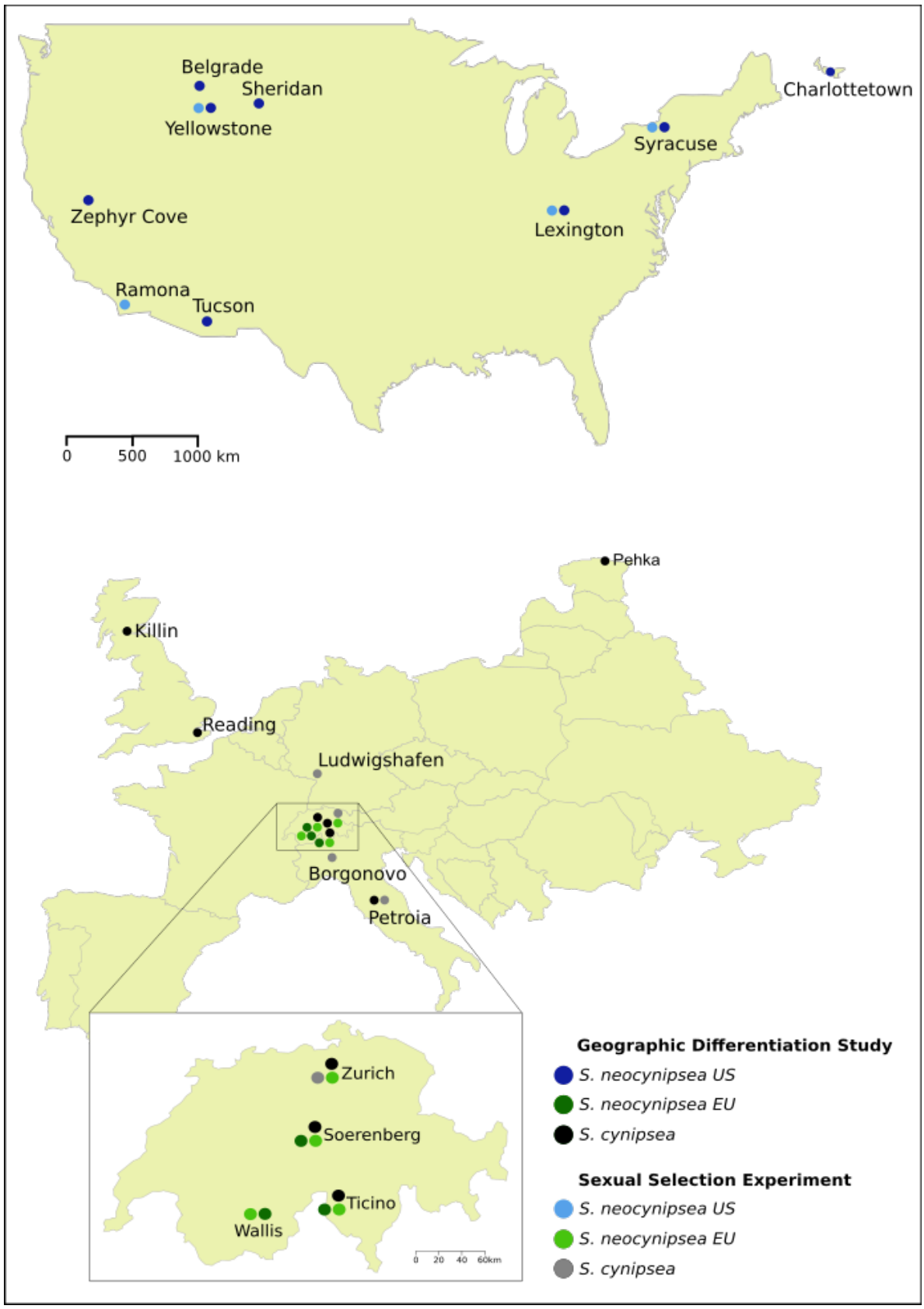

Figure 1: Map of population sampling locations for the geographic Differentiation study (dark colored dots) and for the sexual selection experiment (light colored dots)

Acquisition of morphometric data

Preparation of legs and wings was done in 70% EtOH under a binocular. Both forelegs and wings were removed from the thorax using fine forceps and, after evaporation of the ethanol, embedded in Euparal (C. Roth Gmbh) on a microscopy slide. Attention was paid not to squeeze the specimens at any time, especially when adding the cover slip, and to place all of them in the same orientation, because both, distortion and variation in orientation, are potential sources for major errors in the morphometric analysis. The slides were then placed on a 50ºC heating plate for five minutes to liquefy the artificial resin before samples were dried at room temperature.

The morphometric analysis was performed using landmarks extracted from digitized images photographed with a LeicaDFC490 camera under a Leica MZ12 microscope. In order to describe shape variation of the male foreleg femur, seven landmarks were placed, marking distinct and most probably interspecifically homologous points. In addition, three sliding, evenly spaced, semi-landmarks (Gunz & Mitterroecker, 2013) were placed between landmarks one and two as well as between landmarks six and seven, to measure the curvature of the leg between the fixed landmarks (description and graphical illustration of femur landmarks; *Figure 2*). A set of 16 landmarks was chosen for the wings, marking all vein-node positions in the center of the wing and all vein-nodes at the wing margin (description and graphical illustration of wing landmarks; *Figure 3*), similar to the set of landmarks described by Houle & Mezey (2005).
Landmarks were acquired using the tpsutil (version 1.21.0.1, (Rohlf, 2015)) and tpsdig2 software (version 1.1, (Rohlf, 2006)). The software Past (Hammer et al., 2001) was used to extract the centroid sizes of both morphological traits and to perform a full procrustes transformation. Full Procrustes transformation is a frequently used superimposition method, providing a new set of coordinates for subsequent morphometric analyses. The transformation includes a standardization of scale, which means that the centroid size of each specimen is set to one. Then, centroids of all specimen are translated to the origin of the procrustes coordinate system and rotated to reach minimal sum of squared distance to the mean shape at each landmark (in detail; (Rohlf & Slice, 1990)). Using the Procrustes coordinates, a principal component analysis (PCA), based on covariance matrix, was performed using the package geomorph in the statistical software R (Adams & Otárola-Castillo, 2013; R Development Core Team, 2015). This method is most commonly used for morphometric analyses since it allows graphical illustration of relative shape changes of landmarks and additional analyses of different aspects of shape (Klingenberg & Zaklan, 2000). The PCA was performed on the combined data set, including the flies from the geographic

differentiation sampling and those used in the sexual selection experiment to assure full comparability of the results.

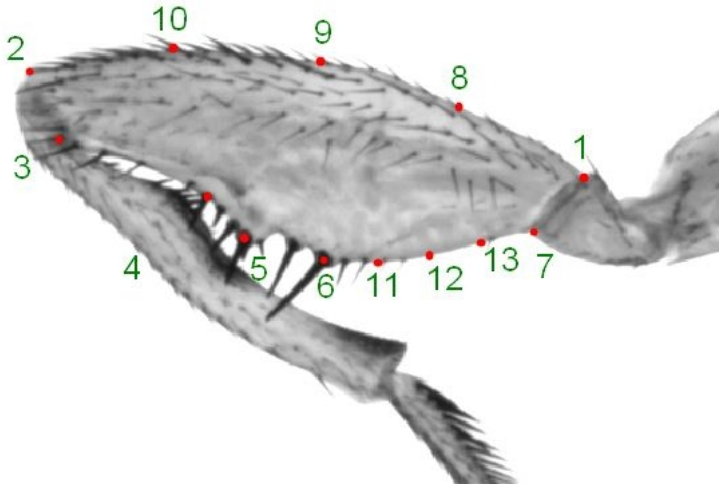

Figure 2: Position of Femur Landmarks; 1. dorsal trochanter – femur transition, 2. dorsal side of femur – tibia joint, 3. ventral side of femur – tibia joint, 4. vertex of the notch, 5. attachment point of 1. main seta, 6. attachment point of second/last (if more than 2) main seta, 7. ventral side of trochanter – femur transition, 8.-10. semilandmarks placed on dorsal margin by means of equal distances between Lm 1 & Lm 2, 11.-13. semilandmarks placed on ventral proximal margin by means of equal distances between Lm 6 & Lm 7

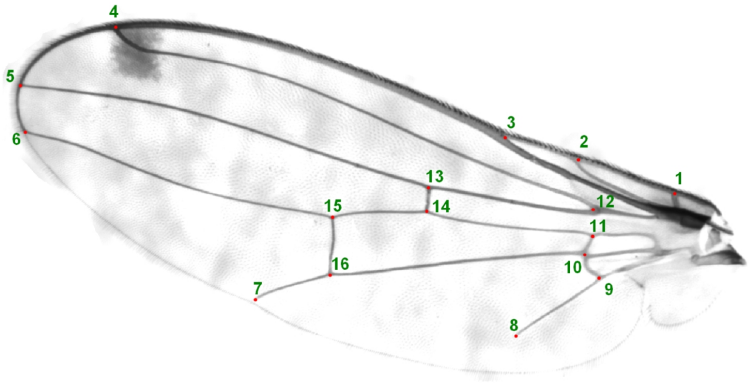

Figure 3: Positions of Wing Landmarks; 1. crossvein – costa, 2. auxiliary vein – costa, 3. first longitudinal vein – costa, 4. second longitudinal vein – radius, 5. third longitudinal vein – radius, 6. fourth longitudinal vein – median, 7. fifth longitudinal vein - cubitus / end of fifth longitudinal vein, 8. end of anal crossvein, 9. anal crossvein - posterior cubital cell, 10. fifth crossvein - second basal cell, 11. fourth crossvein - second basal cell, 12. second - third longitudinal vein, 13. anterior crossvein - third longitudinal vein, 14. anterior crossvein - fourth longitudinal vein, 15. posterior crossvein - fourth longitudinal vein, 16. posterior crossvein - fifth longitudinal vein

Statistical analysis

Prior to any subsequent analysis, the extent to which shape correlates with size was estimated. Two different approaches were used. First, each x and y coordinate was regressed separately on centroid size. The slope was then used to predict the strength and direction of the shape change of each landmark relative to a given change in size (Mitterroecker et al., 2013). This approach allows a graphical illustration of the complete shape change related to any change in size. For graphical visualization R packages geomorph and ggplot2 were used (Wickham, 2009). In a second approach, each principal component (PC) was correlated with centroid size using linear regressions to facilitate the interpretation of the different shape components in a population genetic and sexual selection framework.

Geographic patterns of morphological differentiation across species and populations were analyzed by performing nested linear mixed effects models on the different PCs and the centroid sizes. In these models iso-female lines were nested within populations and populations within lineages (*S. cynipsea*, *S. neocynipsea* North America and Europe). Temperature and lineage were treated as fixed factors and population and iso-female line as random factors. The interaction with temperature was included on all levels. Partial eta squared (η^2) was used to estimate effect size.

To test for morphological clines, centroid sizes and PCs were regressed on latitude based on population mean values. Due to the number and geographic range of sampled populations the latitudinal analysis was only performed for the populations of *S. cynipsea* and North American *S. neocynipsea*.

Sexual selection differentials obtained from the mating experiment were calculated according to Rohner et al. (2016). Standardized trait values (z-scores) of mated and unmated flies were obtained by subtracting the replicate mean trait value from each individual and dividing by the replicate standard deviation. The relative mating success (1 for successful, 0 for unsuccessful, divided by the mean mating success per replicate) was then regressed on the trait value. The resulting regression slope represents the standardized linear sexual selection gradient equal to the selection differential. For each population the selection differentials observed in the different replicates were averaged. In order to test for the statistical significance of the selection differentials, univariate generalized linear models were performed using z-scored trait values as independent variable. In the analyses, lineage, population (nested within lineage) and replicate (nested within population)were used as predictors

and relative mating success as response variable. These analyses were performed based on individual trait values of each male. The interaction between lineage and standardized trait value was included in the analyses to test for differences in the strength and direction of sexual selection among the three lineages. These analyses were done using the statistical software SPSS (version 22.0.0.0).

Results

Shape descriptors of forelegs and wings

Principal components accounting for less than 10% of the total morphological variation were omitted from any further analysis, since they contain mostly random variation and also because it becomes increasingly difficult to understand and visualize the actually described minor effects of changes in shape, considering the overwhelming shifts explained by principal components accounting for higher proportions of variation.

Two major PCs cumulatively explained 71.39% of total shape variation in male femur morphology. PC1 accounted for 51.03% of the shape variation and is primarily related to the width of the femur along the dorso-ventral axis. Flies with negative PC1 scores show wider femurs relative to flies with positive scores (*Figure 4*). PC2, explained 20.36% of the shape variation, and describes predominantly the depth of the notch (LM 4) and the relative positioning of the main setae at the ventral side of the femur (LM 5 & LM 6). Lower scores of PC2 represent a more protruding attachment of the first main seta and a flatter shaped notch (*Figure 5*).

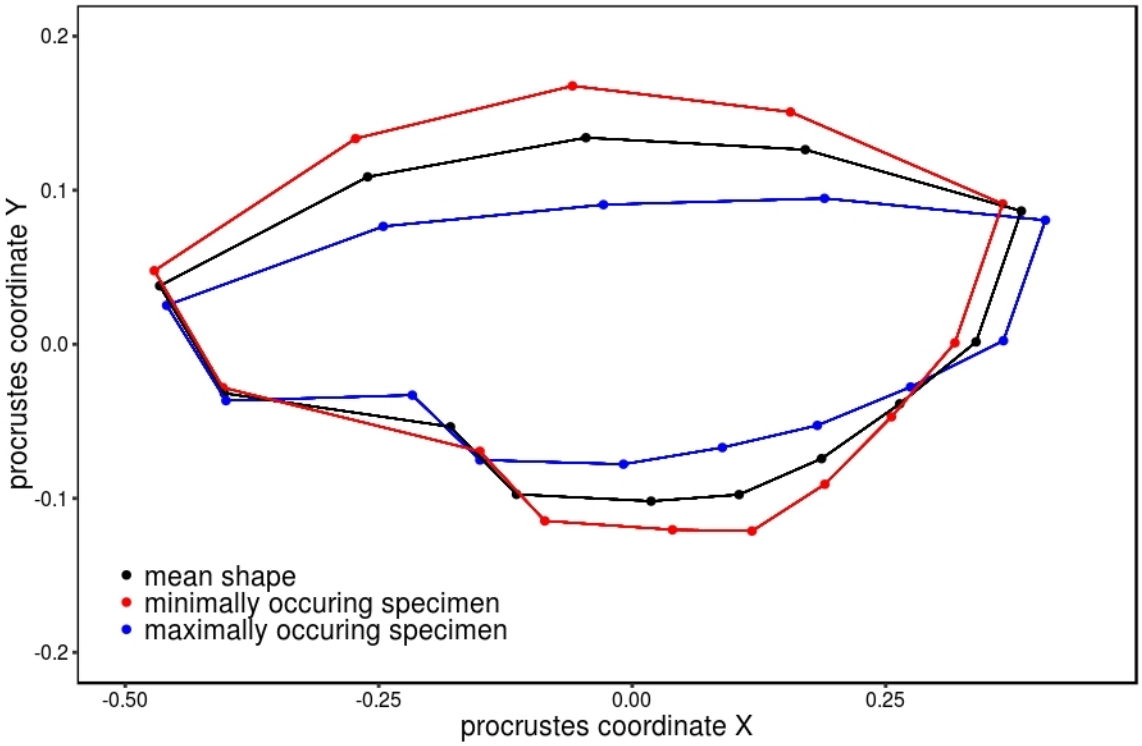

Figure 4: Shape change of the femur described by PC1, illustrated by mean shape (black), the specimen with the lowest (red) and highest (blue) score

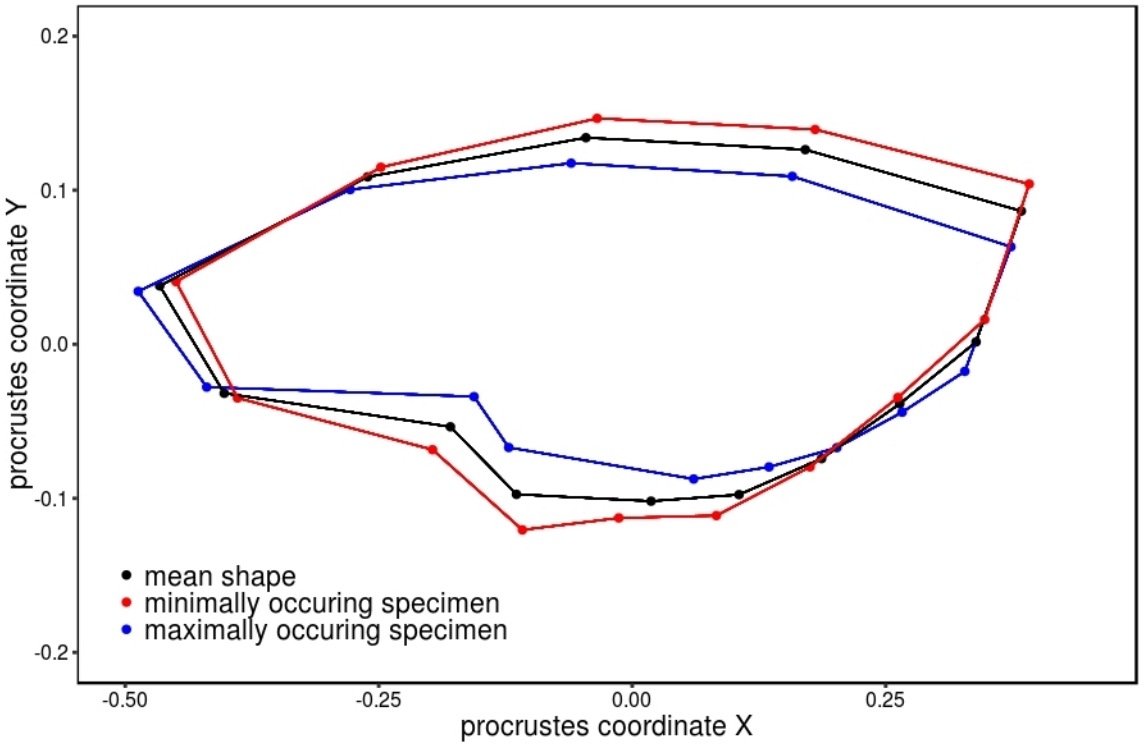

Figure 5: Shape change of the femur described by PC2, illustrated by mean shape (black), the specimen with the lowest (red) and highest (blue) score

Three PCs accounted for 69.83% of the total shape variation in wing morphology. PC1 explains 43.42% of the variation and largely describes the shape of the wing margin. The ratio of wing length to wing width, called wing aspect ratio, is frequently used to describe the overall shape. In the present study, high PC1 scores are related with a low wing aspect ratio. In addition, more elongated wings (high wing aspect ratio) are associated with a shift of the anterior and posterior cross-vein towards the base of the wing, while more roundish wings tend to have both central cross-veins more distally located (*Figure 6*). PC2, accounting for 13.43% of the total variation, characterizes a convergence of the anterior (LM 13 & LM 14) and the posterior cross-vein (LM 15 & LM 16). Furthermore, flies with high value on PC2 have wider wings at the 3rd posterior cell, caused by a more proximal positioning of the 5th longitudinal vein (LM 16 & LM 7) and a slight shift of the anterior wing margin (*Figure 7*). PC3, which explained 12.98%, is largely related to wing width. Compared to PC2, which shifts LM 7 and LM 16 in proximal direction, PC3 is associated with a shift in posterior direction. Furthermore high values on PC3 are related to a shift of the anterior cross-vein towards the base of the wing while low values displace it to a more distal position (*Figure 8*).

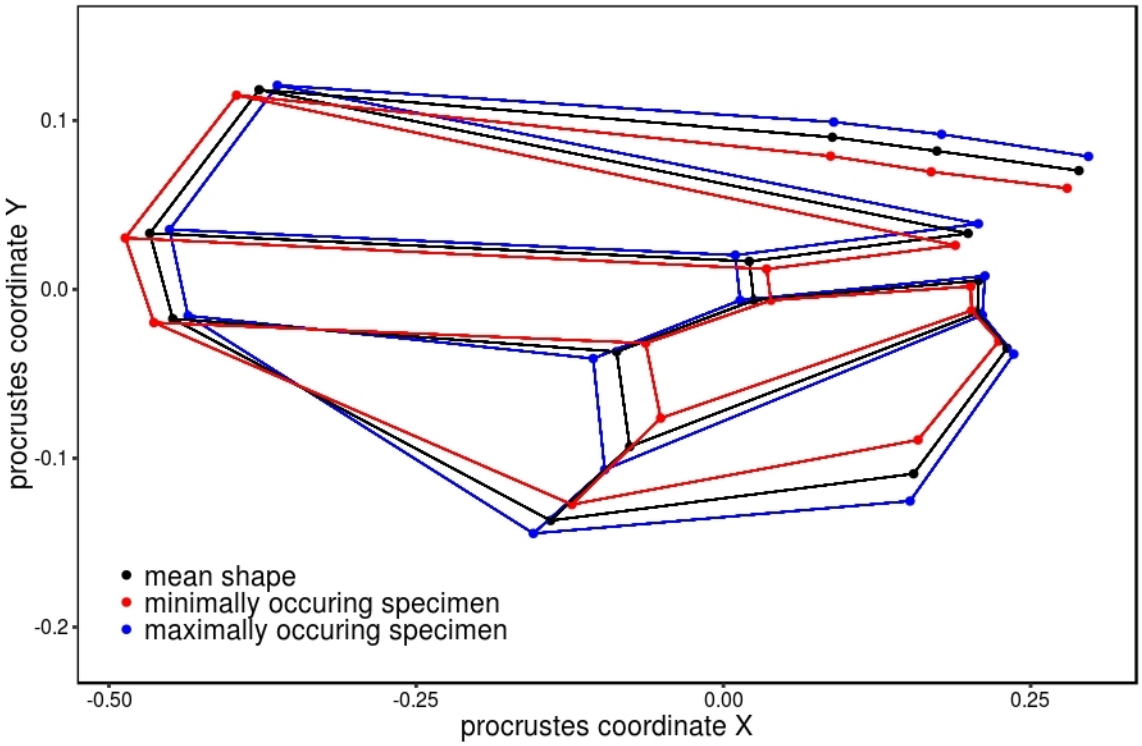

Figure 6: Shape change of the wing described by PC1, illustrated by mean shape (black), the specimen with the lowest (red) and highest (blue) score

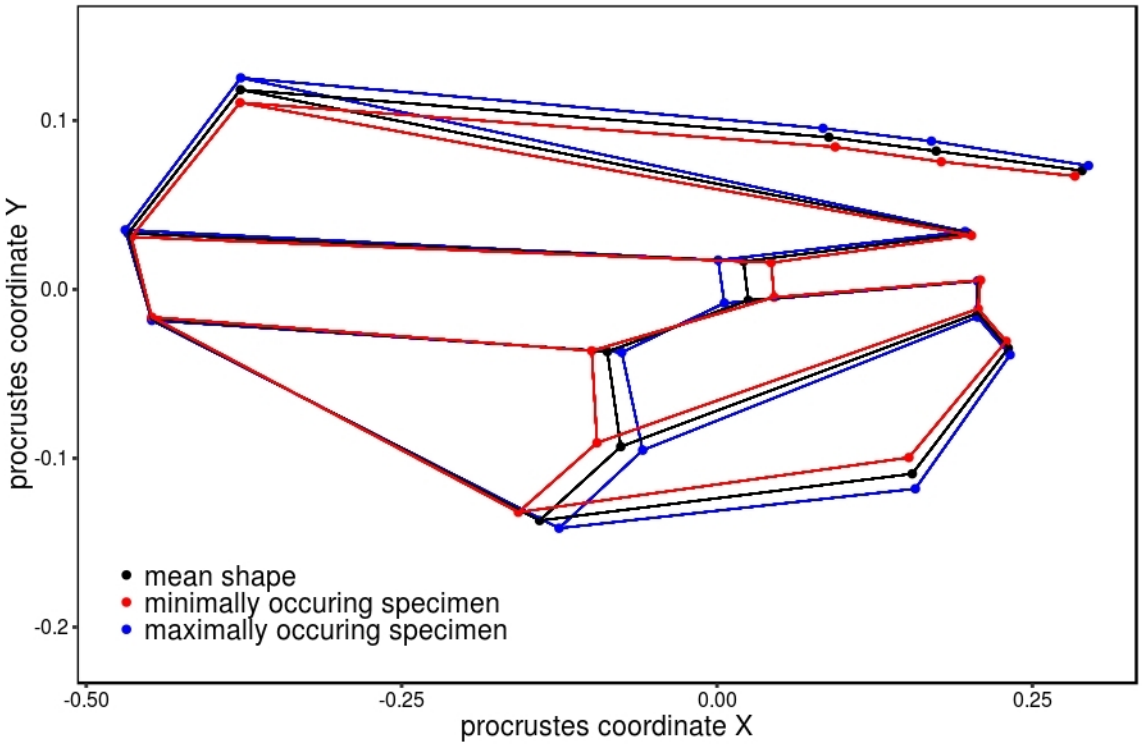

Figure 7: Shape change of the wing described by PC2, illustrated by mean shape (black), the specimen with the lowest (red) and highest (blue) score

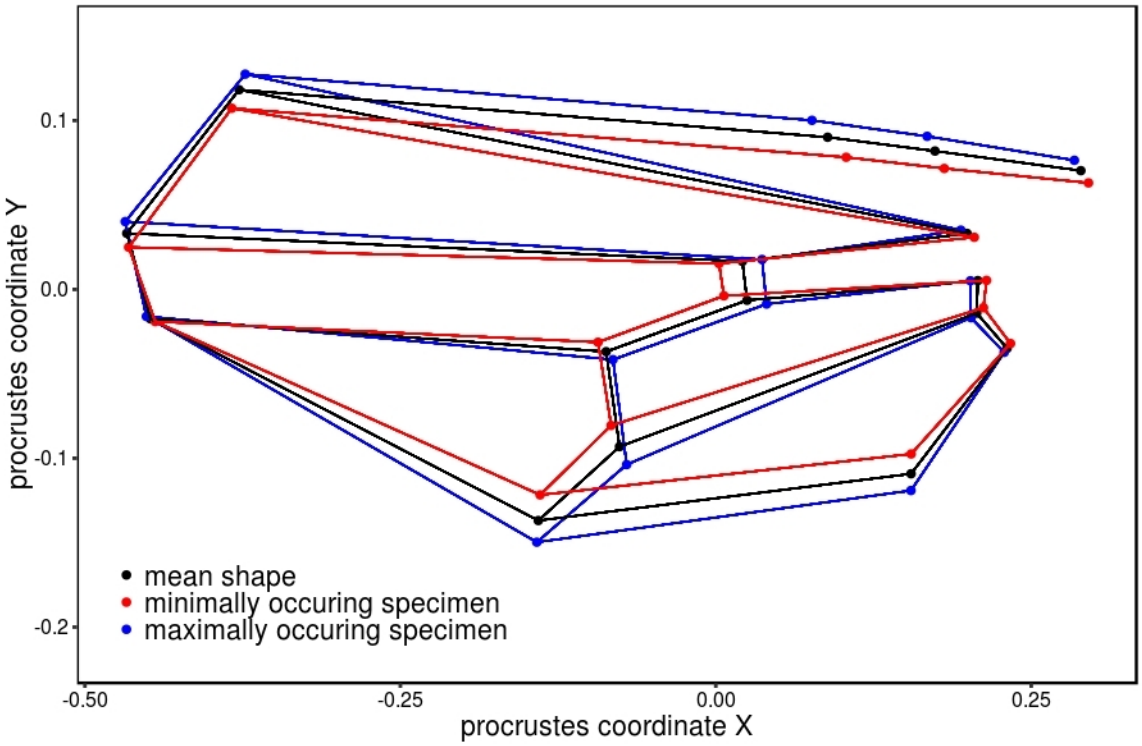

Figure 8: Shape change of the wing described by PC3, illustrated by mean shape (black), the specimen with the lowest (red) and highest (blue) score

Allometric relationships of foreleg and wing shape

Two approaches were used to investigate the allometric relationship between size and shape aspects of the respective traits. In a first approach the overall change in shape correlated with changing size is described and compared between the three lineages. In a second approach, the extent to which shape aspects, as described by principal components, depend on size is estimated and compared between lineages for consideration in the interpretation of further analysis.

Overall, the shape of large sized femurs was much wider compared to the shape of small femurs. The allometric slope was of similar magnitude in North American (*Figure 9*) and European populations of *S. neocynipsea* (*Figure 10*) and slightly weaker in *S. cynipsea* (*Figure 11*). The three lineages further revealed differences in the relative positioning of the attachment of the main setae at the ventral side of the femur, which strongly co-varied with centroid size in North American and European populations of *S. neocynipsea*, but to a much lesser extent in *S. cynipsea*. Additionally, the notch (LM 4) indicated a strong x-directional shift in the European *S. neocynipsea* lineage. Linear regressions of the different PCs on centroid size yielded a similar picture (*Table 1*). PC1 strongly correlated with femur size in all lineages. On average 70% of the variation of *S. neocynipsea* in PC1 can be explained by centroid size, while in the *S. cynipsea* lineage only 40% of the variation is attributable to femur size. Also, PC2 significantly correlates with size in all lineages. This relationship is strongest in the European *S. neocynipsea* lineage.

	S. neocynipsea US		
	R^2	slope	p-value
PC1	0.63	-7.11	**<0.01**
PC2	0.18	-2.65	**<0.01**

	S. neocynipsea EU		
	R^2	slope	p-value
PC1	0.76	-5.53	**<0.01**
PC2	0.37	-3.27	**<0.01**

	S. cynipsea		
	R^2	slope	p-value
PC1	0.40	-4.28	**<0.01**
PC2	0.12	-2.26	**<0.01**

Table 1: Regression of femur-shape components on Centroid size

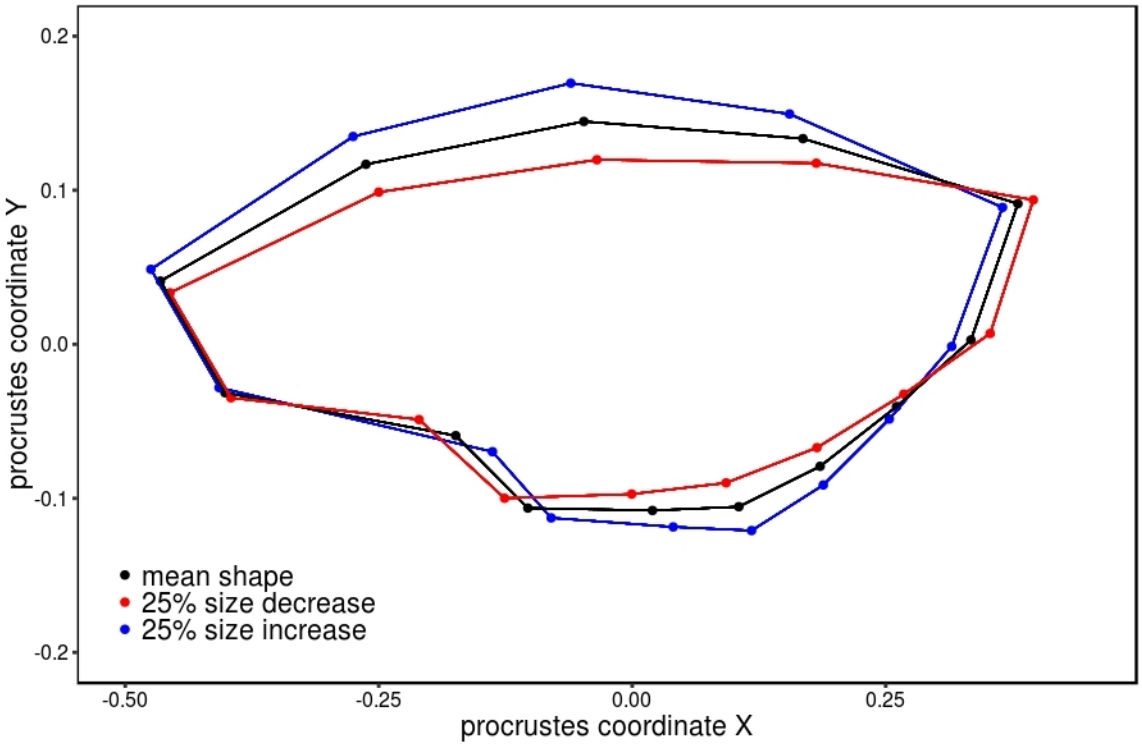

Figure 9: Size-dependent shape change of the femur in North American S. neocynipsea, mean shape and shape of an extrapolated 25% bigger (blue) and 25% smaller (red) individual

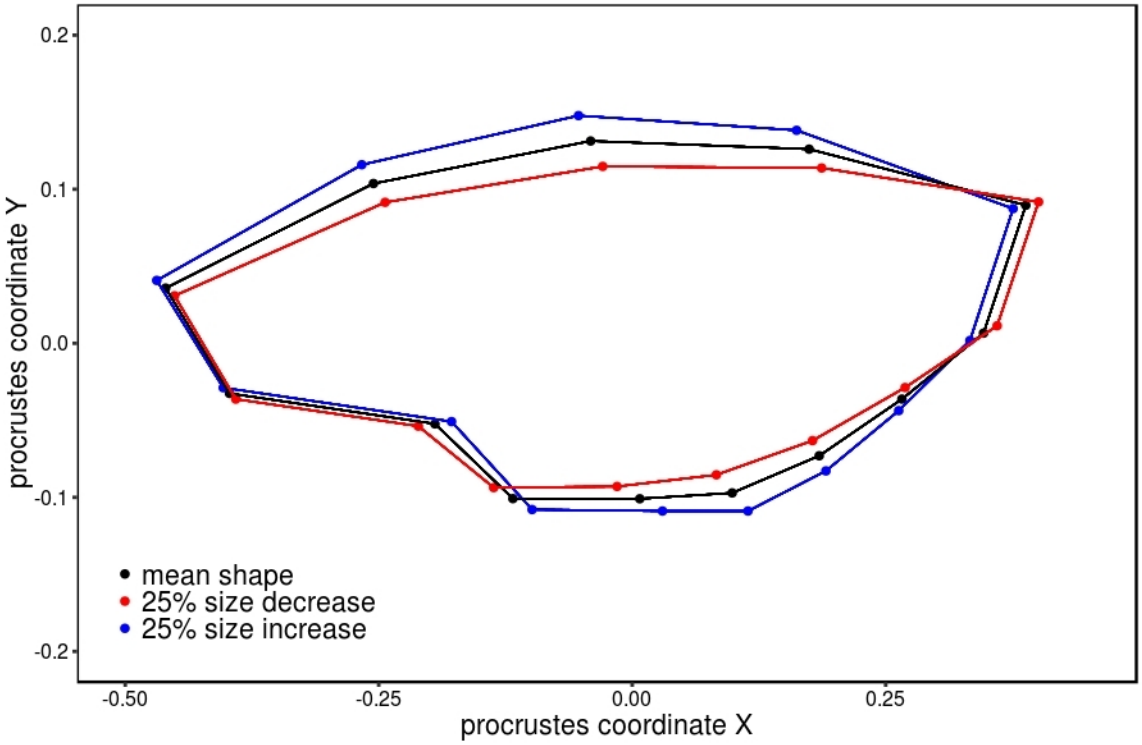

Figure 10: Size-dependent shape change of the femur in European S. neocynipsea, mean shape and shape of an extrapolated 25% bigger (blue) and 25% smaller (red) individual

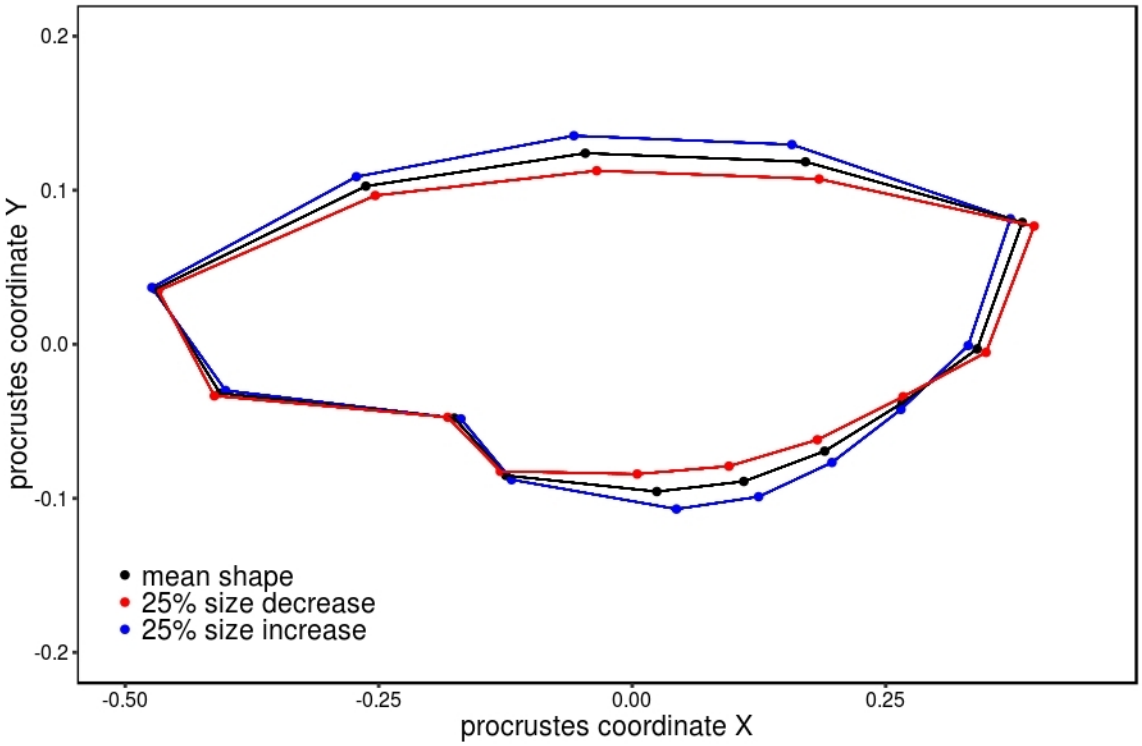

Figure 11: Size-dependent shape change of the femur in S. cynipsea, mean shape and shape of an extrapolated 25% bigger (blue) and 25% smaller (red) individual

In contrast to femur shape, the allometric components of the PCs extracted for wing shape were much smaller, albeit statistically significant (*Table 2*). In general, larger wings tended to be more roundish with the anterior and posterior cross-veins situated more central in the wing across all lineages (*Figure 12, 13, 14*). However, *S. neocynipsea* indicated more size dependent variation in respect to the size of the 3rd posterior cell, mostly caused by a shift of the end of the anal cross-vein (LM 8). Similar to femur morphology, PC1 significantly depends on size in all lineages, although the relationship was rather weak. The proportion of variation explained by size is higher in *S. neocynipsea* than *S. cynipsea*, and within *S. neocynipsea* considerably higher in European than American populations. The relationship is close to isometry in all lineages and mostly caused by the relative shortening of the wing in larger individuals and an increase in size of the 3rd posterior cell relative to other wing cells. PC2 only indicated a significant (isometric) relationship with wing size in American flies. The analysis of PC3 again resulted in a significant allometric relationship in all lineages, but these relationships were found to be very weak.

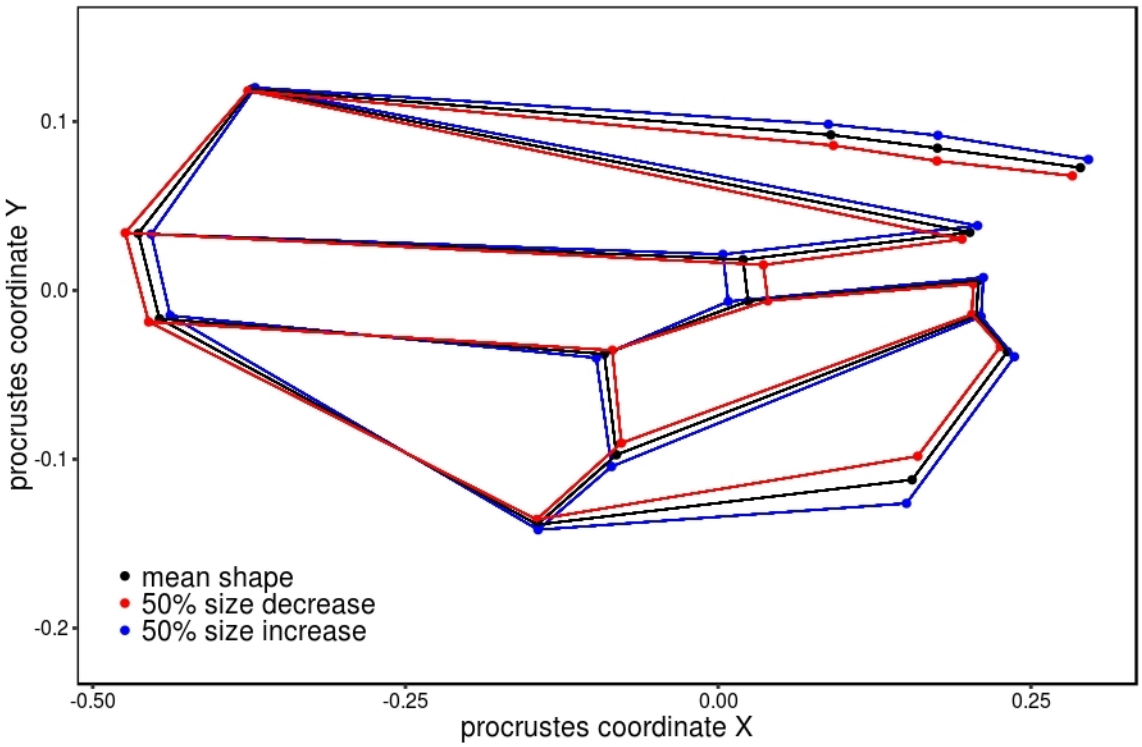

Figure 12: Size-dependent shape change of the wing in North American S. neocynipsea, mean shape and shape of an extrapolated 50% bigger (blue) and 50% smaller (red) individual

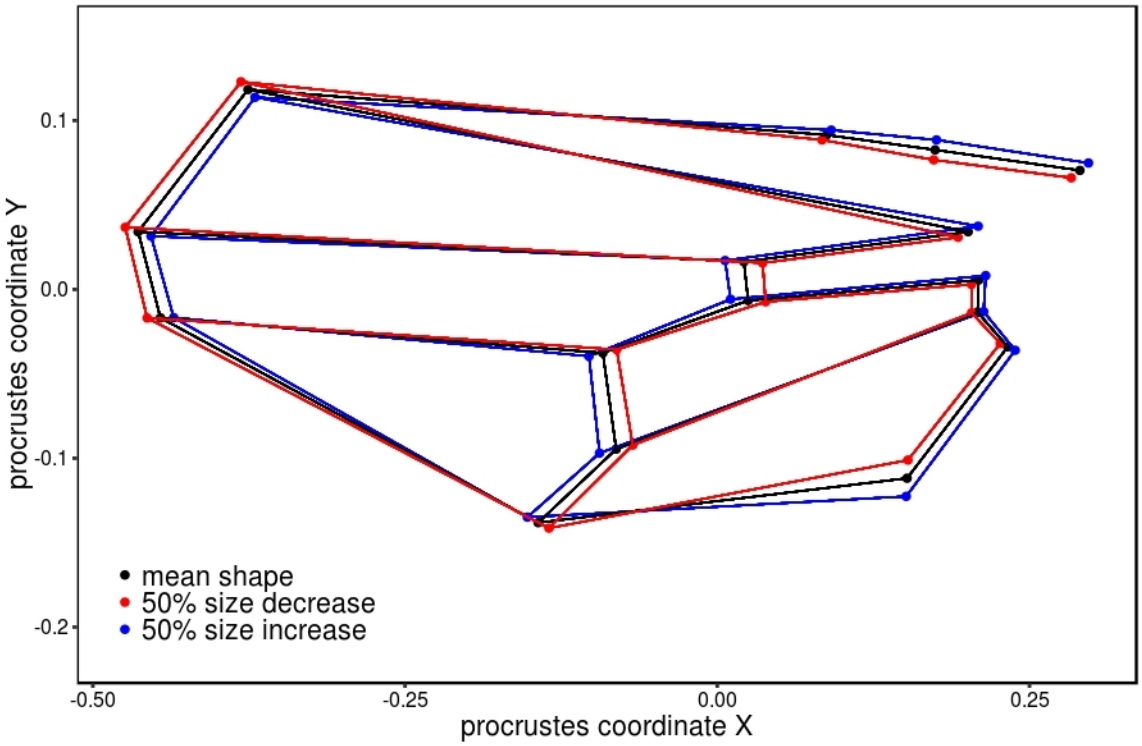

Figure 13: Size-dependent shape change of the wing in European S. neocynipsea, mean shape and shape of an extrapolated 50% bigger (blue) and 50% smaller (red) individual

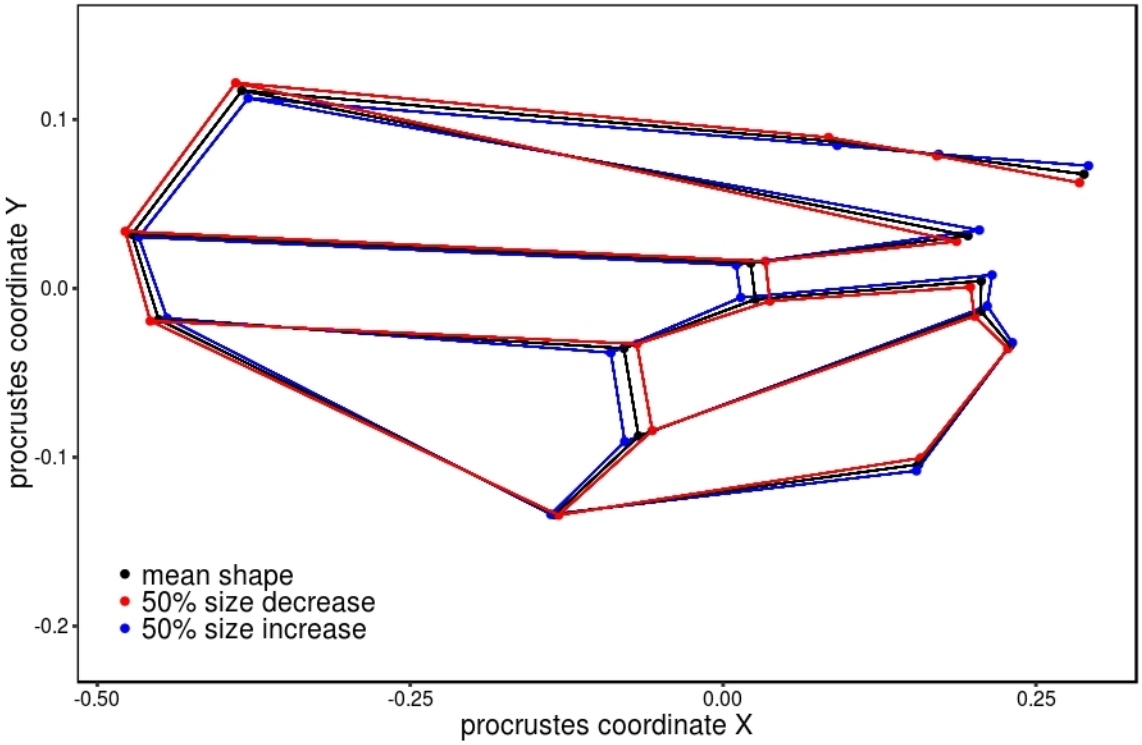

Figure 14: Size-dependent shape change of the wing in S. cynipsea, mean shape and shape of an extrapolated 25% bigger (blue) and 25% smaller (red) individual

	S. neocynipsea US		
	R^2	slope	p-value
PC1	0.18	0.90	**<0.01**
PC2	0.12	-0.96	**<0.01**
PC3	0.02	0.30	0.01

	S. neocynipsea EU		
	R^2	slope	p-value
PC1	0.34	1.06	**<0.01**
PC2	0.01	-0.22	0.10
PC3	0.14	0.88	**<0.01**

	S. cynipsea		
	R^2	slope	p-value
PC1	0.10	0.79	**<0.01**
PC2	0.00	-0.07	0.69
PC3	0.06	0.94	**<0.01**

Table 2: Regression of wing-shape components on Centroid size

Geographic patterns of morphological differentiation

Nested linear mixed effect models revealed significant quantitative genetic differentiation in male foreleg morphology between and within lineages (*Table 4*). In agreement with earlier population based studies of body size variation in the study species (Rohner et al., 2016), North American populations of *S. neocynipsea* had larger sized femurs compared to European *S. neocynipsea* and *S. cynipsea* (*Figure 15*). As illustrated in *Figure 16*, PC1 clearly separates North American *S. neocynipsea* from the other two lineages. PC2 separated *S. cynipsea* from *S. neocynipsea* on both continents. Significant morphological differentiation was also evident within continents. Population main effects on PC1 and PC2 were both significant.

For the wings, PC1 contained a strong phylogenetic signal, supporting the taxonomic status of the species. *S. cynipsea* was found to have more elongated wings (as indicated by negative scores of PC1) relative to *S. neocynipsea*, which evolved more roundish wings (as indicated by positive scores of PC1) (*Figure 17*). PC2 was only marginally differentiated among the three lineages such that *S. cynipsea* showed on average slightly higher PC2 scores compared to populations of *S. neocynipsea* from both continents. PC3 and centroid size did not indicate any lineage differentiation.

Similar results are obtained from geographic patterns within lineages, where populations were only significantly differentiated in foreleg but not in wing morphology. Except wing centroid size, which indicated weak, albeit statically significant, differences among lineages. Iso-female line effects, as main effect or in interaction with temperature, were highly significant throughout, indicating substantial standing genetic variance encoding for foreleg and wing morphology.

In order to explore within-lineage patterns of geographic differentiation in more detail, tests for latitudinal clines across North American populations of *S. neocynipsea* and populations of *S. cynipsea* were performed. A significant latitudinal cline in femur size (but not wing size) was found in North America, such that flies from northern populations evolved larger femurs than southern populations (*Figure 18*). Neither femur shape nor wing shape exhibited latitudinal clines across North American *S. neocynipsea* or European *S. cynipsea* populations (all $P>0.05$).

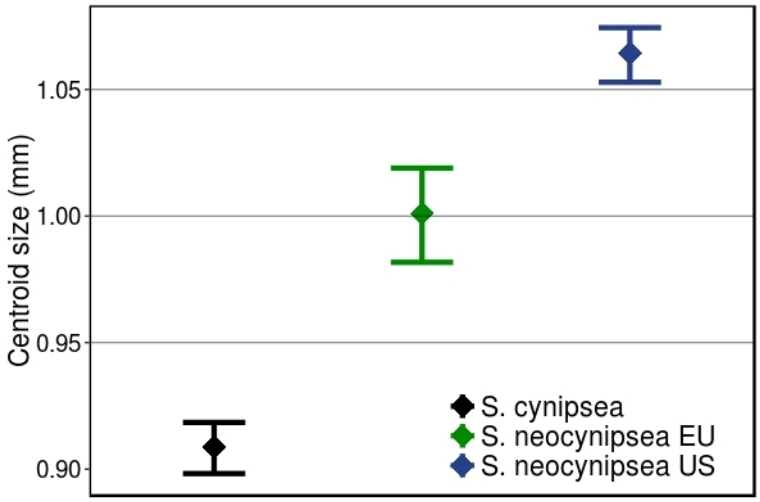

Figure 15: Mean Femur Centroid size with 95% CI

Temperature-dependent plasticity

Two temperature regimes were applied in the common garden experiment, to address phenotypic plasticity of the investigated traits. Contrary to the expectations, temperature effects were in general quite weak (*Table 3, 4*). Nevertheless, temperature influenced femur size, but not wing size, such that flies raised at 18ºC developed larger femurs compared to flies which were raised at 24°C. In addition, significant temperature by population interactions, affecting centroid sizes of foreleg and wing size, were found, suggesting a genetic basis of phenotypic plasticity.

		PC1			PC2			CentroidSize		
	df	F	p	η^2	F	p	η^2	F	p	η^2
Lineage	1	7.20	**0.01**	0.49	38.10	**<0.01**	0.84	17.10	**<0.01**	0.70
Temperature	2	3.23	0.09	0.17	2.43	0.14	0.13	5.04	**0.04**	0.25
L*T	2	0.42	0.67	0.05	1.80	0.20	0.19	1.82	0.20	0.20
Population(L)	15	7.39	**<0.01**	0.84	3.55	**0.01**	0.76	3.48	**<0.01**	0.69
Line(P(L))	122	1.77	**<0.01**	0.74	1.48	**0.03**	0.71	2.48	**<0.01**	0.80
P(L)*T	14	1.84	**0.05**	0.28	1.58	0.11	0.25	3.87	**<0.01**	0.45
Li(P(L))*T	70	1.77	**<0.01**	0.26	1.65	**<0.01**	0.25	1.61	**<0.01**	0.24
Error	353									

Table 3: Nested linear mixed effects model; Femur

		PC1			PC2			PC3			CentroidSize		
	df	F	p	η^2	F	p	η^2	F	p	η^2	F	p	η^2
Lineage	1	31.38	**<0.01**	0.81	3.76	**0.05**	0.35	1.29	0.30	0.15	1.72	0.21	0.11
Temperature	2	1.46	0.24	0.09	1.29	0.27	0.07	1.98	0.18	0.12	2.61	0.11	0.26
L*T	2	0.13	0.88	0.02	2.44	0.12	0.24	0.33	0.72	0.04	1.48	0.26	0.17
Population(L)	15	2.71	0.01	0.58	1.40	0.21	0.40	1.65	0.15	0.57	2.72	**0.03**	0.73
Line(P(L))	120	2.26	**<0.01**	0.78	2.29	**<0.01**	0.78	1.91	**<0.01**	0.75	2.08	**<0.01**	0.77
P(L)*T	14	1.64	0.09	0.25	1.49	0.14	0.24	4.46	**<0.01**	0.48	22.25	**<0.01**	0.82
Li(P(L))*T	70	1.60	**<0.01**	0.24	1.22	0.13	0.19	1.44	**0.02**	0.22	1.97	**<0.01**	0.28
Error	362												

Table 4: Nested linear mixed effects model; Wings

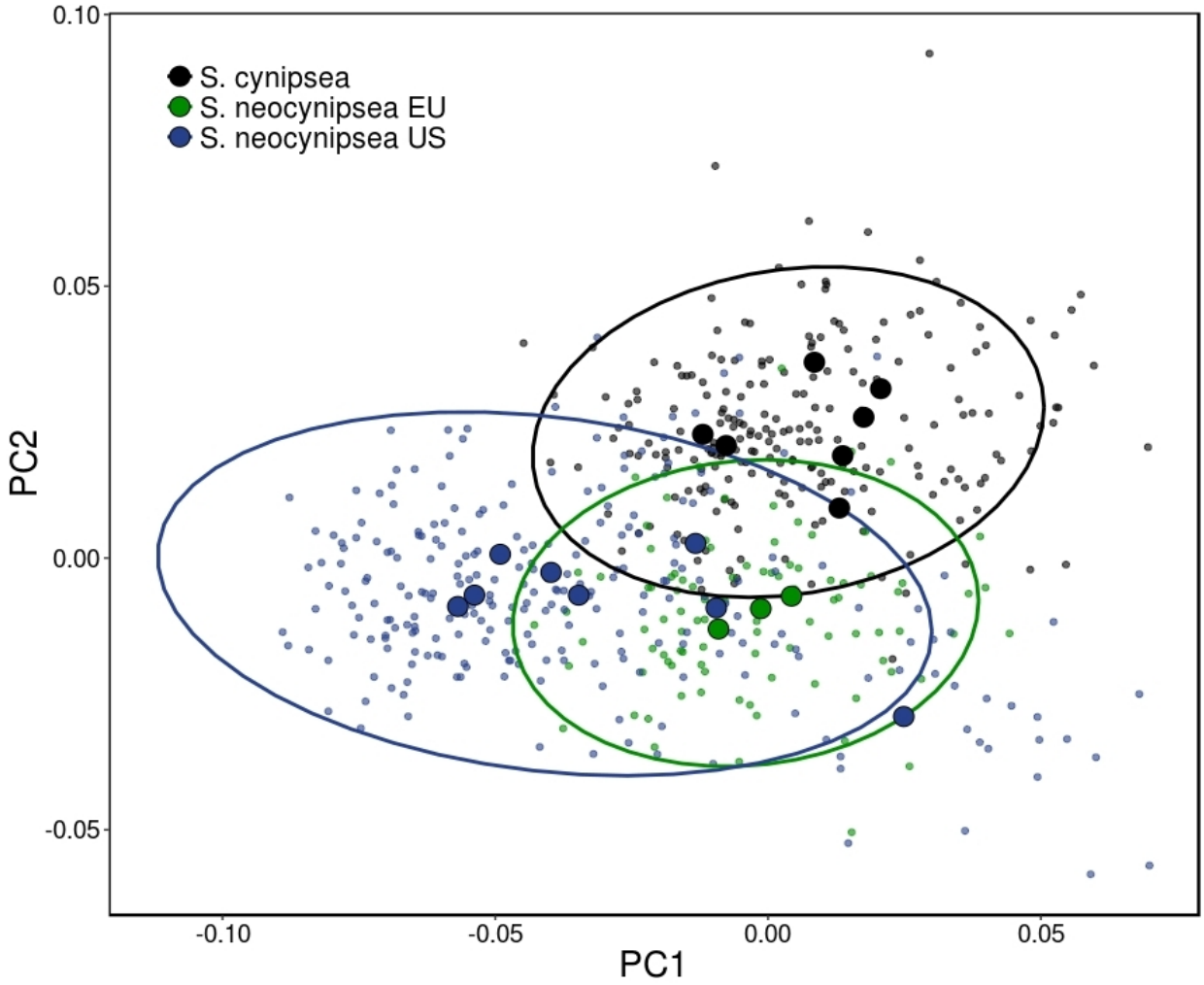

Figure 16: Morphological differentiation of the Femur on PC1 and PC2 including 95% ellipses for lineages and population means as bold points

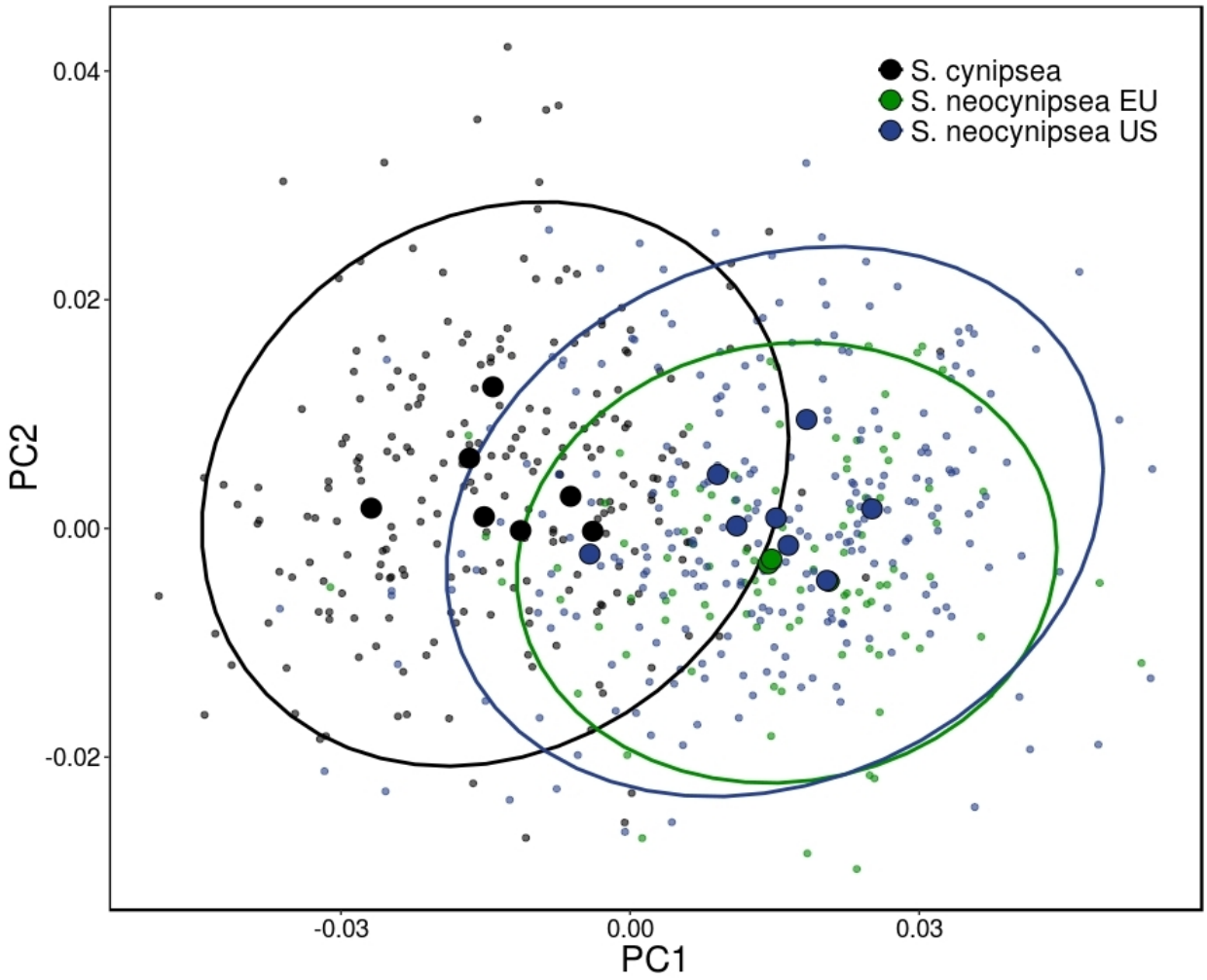

Figure 17: Morphological differentiation of the Wing on PC1 and PC2 including 95% ellipses for lineages and population means as bold points

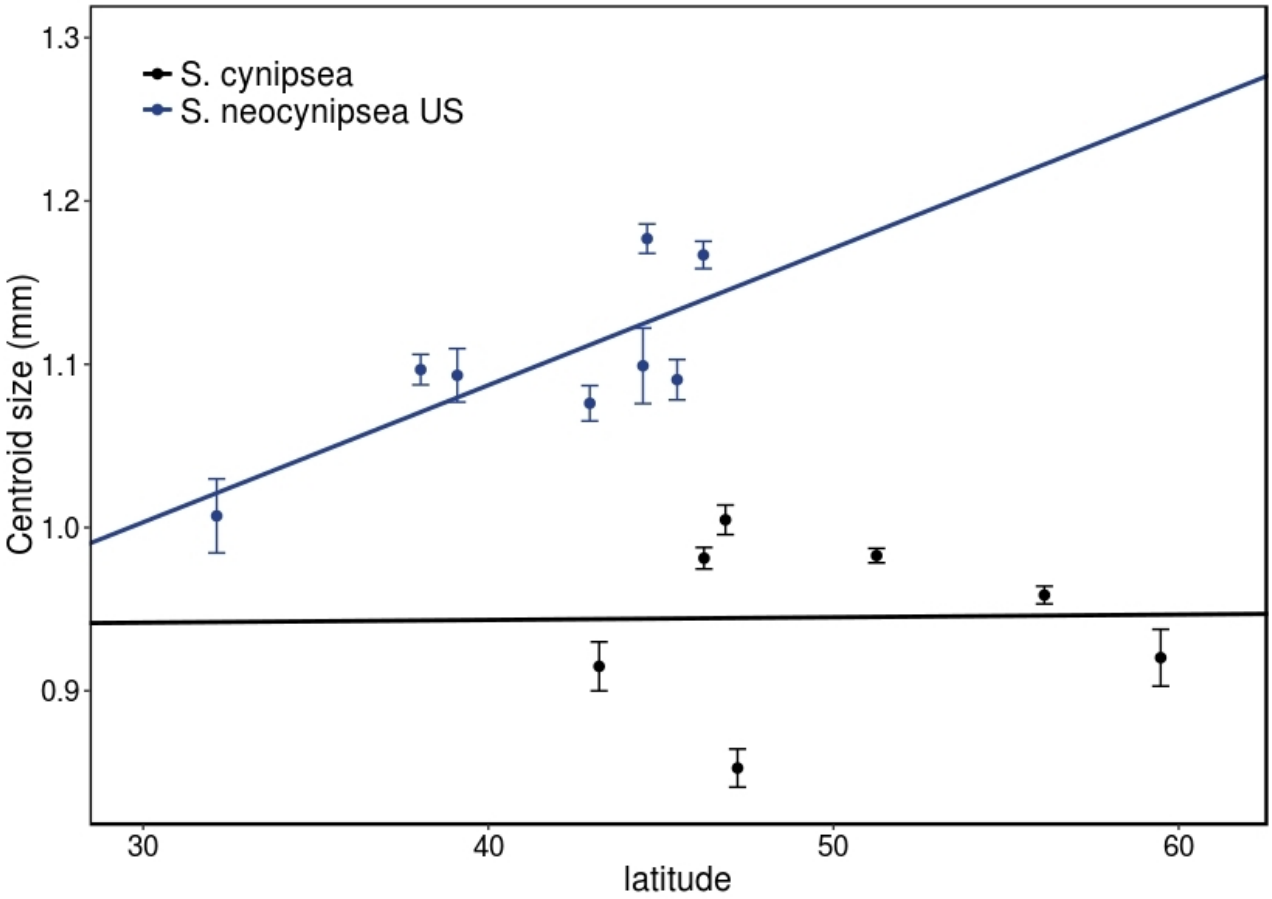

Figure 18: Latitudinal clines of femur size of North American S. neocynipsea (R^2 = 0.58; p = 0.02) and European S. cynipsea (R^2 < 0.001; p = 0.97)

Sexual selection on foreleg and wing morphology

The sexual selection experiment revealed, that the size of the male femur is positively selected for in all lineages, with the strongest selection differentials found in American populations of *S. neocynipsea* (*Figure 19*). Also, the mating experiments provided evidence for sexual selection acting on femur shape (*Table 5*). Flies with negative PC1 scores, which mainly reflect broader and more compact femurs, tended to achieve higher mating success in all lineages. PC2 indicated a disruptive pattern of selection. While in European *S. neocynipse*a less protruding main setae and a deeper notch are advantageous, the opposite is the case in the remaining lineages.

There is also experimental evidence for sexual selection acting on wing size and shape (*Figure 19*, *Table 6*). The strength and direction of selection on wing size varied among lineages. While in *S. cynipsea* the experiment resulted in a slightly negative average selection coefficient, those for *S. neocynipsea* were positive in sign. Significant selection differentials were also detected for wing shape. In all lineages, flies with more roundish than elongated wings (indicated by PC1) were found to have higher mating success. PC2 indicated significant differences in sexual selection strength and direction. In European *S. neocynipsea* populations, flies with high PC2 scores were more successful in gaining matings, while the direction altered in the other two lineages. PC3 was only affected by selection across American populations of *S. neocynipsea*. All measured selection differentials of both traits were found to be less variable across the American populations of *S. neocynipsea* compared to the other two lineages.

		PC1	PC2	Size
	df	p	p	p
Lineage	2	1	1	1
Population(L)	9	1	1	1
Replicate(P(L))	23	1	1	1
Trait	1	**<0.01**	0.16	0.29
T*L	*2*	**<0.01**	**<0.01**	**0.04**
T*P(L)	9	**<0.01**	**<0.01**	**<0.01**

Table 5: Univariate generalized linear models for sexual selection differentials of the Femur

		PC1	PC2	PC3	Size
	df	p	p	p	p
Lineage	2	1	1	1	1
Population(L)	9	1	1	1	1
Replicate(P(L))	23	1	1	1	1
Trait	1	**<0.01**	0.51	0.21	0.26
T*L	2	0.19	**<0.01**	**0.01**	**<0.01**
T*P(L)	9	**<0.01**	**<0.01**	**<0.01**	**<0.01**

Table 6: Univariate generalized linear models for sexual selection differentials of the Wings

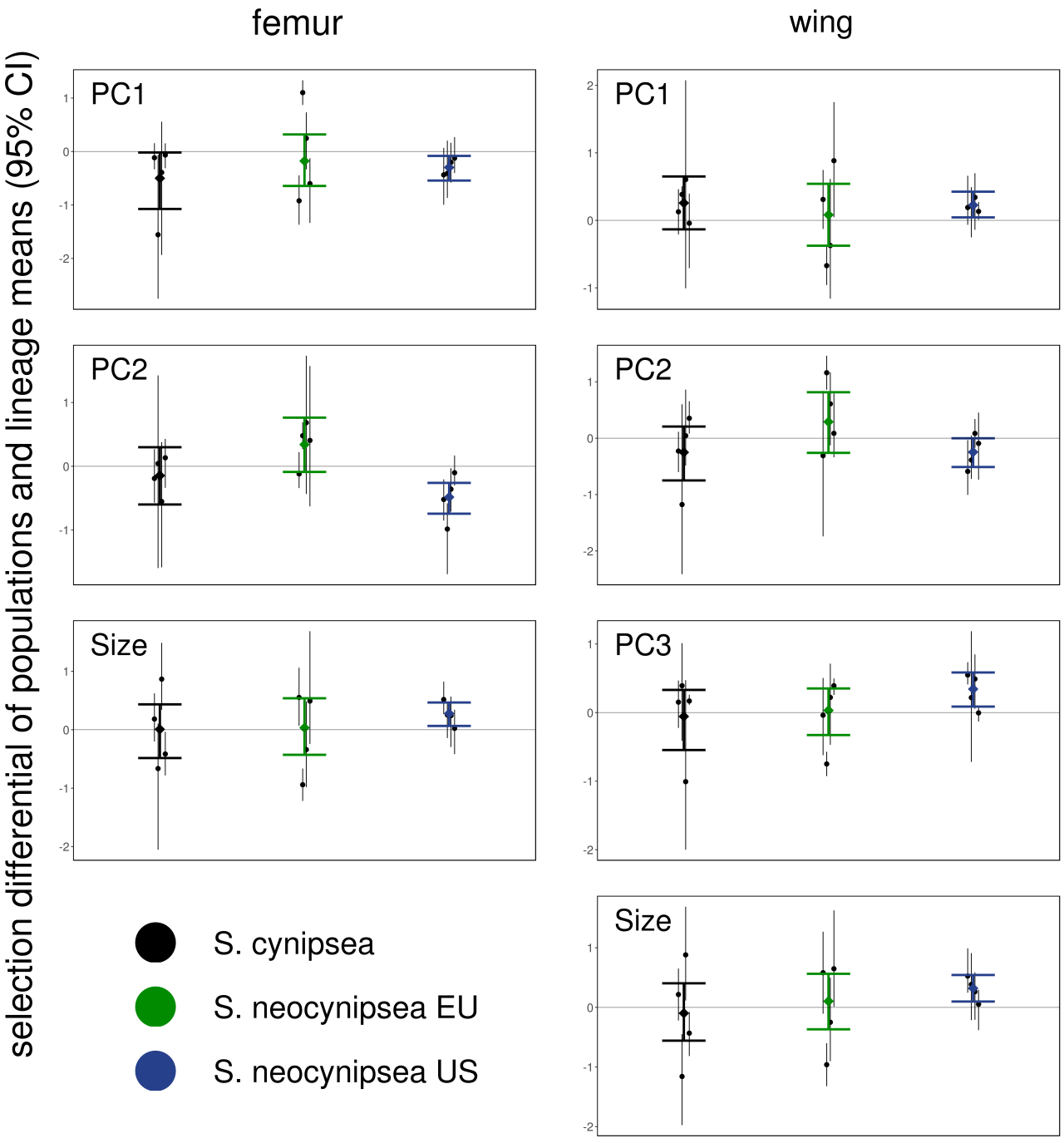

Figure 19: Lineage and population mean sexual selection differentials with 95% CI

Discussion

In the present study of two closely related Sepsid fly species, I compared geographic patterns of quantitative genetic differentiation of an exaggerated male trait (the armored foreleg femur) and of geometric wing morphology. I also assessed the contribution of sexual selection to trait divergence of legs and wings. I quantified selection differentials of allopatric (American) and sympatric (European) populations of *S. neocynipsea* as well as the selection differentials of European populations of *S. cynipsea*. Morphometric analyses revealed stronger divergence in male foreleg compared to wing morphology among both, species and populations. Furthermore, while wing shape contained a strong phylogenetic signal and showed little trait-size related shape variation, the phylogenetic signal with regard to femur shape was outweighed by a phylogeographic signal and trait-size relations of shape aspects were much stronger. Finally, replicated mating experiments revealed significant sexual selection acting on male forelegs, but also on wings.
In the following, I first discuss the role of sexual selection and reproductive character displacement in shaping phylogeographic patterns of morphological differentiation. Secondly, I debate the influence of mating systems and modes of sexual selection on trait evolution. Third, I take a closer look at the implications of size- and condition-dependency of trait morphology and then turn focus to alternative evolutionary forces, that might have contributed to the diversification of the armored forelegs and the wings.

Sexual selection and reproductive character displacement

Sexual selection is considered to be a main driving force behind the evolution of exaggerated male secondary traits (Darwin, 1871; Lande, 1981; Andersson, 1994). However, alternative processes, such as reproductive character displacement, making other predictions about rates of morphological change in time and space, may also play a role. As opposed to sexual selection due to competition or conflict, which acts more or less continuously on a given trait, character displacement predicts rapid trait diversification only during early stages of speciation in geographic areas of co-existence (Servedio & Noor, 2003). If character displacement was important in shaping the male foreleg femur, I would expect stronger differentiation in European populations of *S. neocynipsea*, where they occur in sympatry with *S. cynipsea*, compared to allopatric American populations of *S. neocynipsea*. This expectation was not met by the results. Given the low degree of molecular divergence between and within species as revealed by COI sequences and microsatellite data

(Giesen, unpublished data), significant population differentiation within species in combination with the relatively weak phylogenetic signal among lineages can be better explained by continuous sexual selection acting on the trait. This hypothesis is strongly supported by the laboratory experiments presented in this study, demonstrating significant sexual selection on femur morphology in all lineages regardless of their origin.

Although sexual selection appears to be the main driving force behind the evolutionary diversification of male femur shape, the present data do not exclude the involvement of character displacement. The divergent selection gradients, detected for PC2 between European *S. cynipsea* and *S. neocynipsea*, are congruent with reinforcement by character displacement. Theory further suggests, that reproductive character displacement should be asymmetrical if sympatric species occur in different abundance, in particular, that stronger displacement is expected in the less abundant species, because interspecific encounters are more likely (Servedio & Kirkpatrick, 1997). In Europe, *S. neocynipsea* is relatively rare and largely restricted to high altitudes. Yet, even at high altitudes *S. cynipsea* is the dominating species (pers. obs., (Pont & Meier, 2002). This leads to the question, if the positive selection differentials detected for PC2 only in European *S. neocynipsea*, but not in the other lineages (where selection differentials were negative), reflect the different demands for character displacement in areas of sympatry as opposed to mate choice in geographic areas of allopatry. Mating experiments within and between species would be highly informative on whether European *S. neocynipsea* females indeed show higher resistance to copulate if mounted and grasped by males of the other lineages.

Modes of sexual selection

Comparative studies of Sepsid flies indicate great variation in mating systems leading to different intensities of sexual selection acting on specific male traits. For instance, Puniamoorthy et. al. (2012) showed, that a shift in the mating system of *S. punctum* is associated with a continental reversal of sexual size dimorphism. In European populations, where resource defense polygyny is dominating the mating system, males are larger than females, while in American populations, in which female choice is most important, males are smaller. Similar conclusions have been reached for the reversal in sexual size dimorphism between American and European populations of *S. neocynipsea* (Rohner et al., 2016). My results, indicating that sexual selection on femur size is stronger in the American than European populations of *S. neocynipsea*, are consistent with these earlier studies and also with the relatively weak sexual selection on male body size found in *S. cynipsea*, a species in which scramble competition and female choice are dominating the mating

system (Blanckenhorn et al., 2004). As proposed by Eberhard (2005), female choice can be highly dependent on the stimulation of sensory organs at the female wing base by male forelegs. He comes to the conclusion, that in some sepsids this species-specific stimulation is inducing the mating process (Eberhard, 2001, 2002a). As yet, no studies in Sepsid species are available addressing the exact role of sexual selection for shape divergence of male secondary sexual traits. Regarding femur shape, the only results comparable to this study were found by Blanckenhorn et al. (2004), reporting that sexual selection favors a shorter femur base in *S. cynipsea*, which is comparable with the reduction of the distance between landmark 6 and 7 relative to femur size as captured by PC1. My findings highlight the advantage of exact morphometric measures over linear measures (Rohlf & Marcus, 1993) to detect sexual selection.

The mating experiments also reveal a role of sexual selection acting on wing shape in Sepsid flies. Studies, using *Drosophila melanogaster,* not only demonstrated that wing shape influences flight performance (Ray et al., 2016) and may evolve adaptively in response to environmental gradients (Gilchrist et al., 2001), but also suggest sexual selection to favor males with more elongated wings (Menezes et al., 2013). By contrast, the results found in this study indicate, that males with relatively wide wings are favored by selection. While Drosophila males mainly use wings for acoustic signaling through courtship songs (Greenspan & Ferveur, 2000; Markow & Grady, 2005), in Sepsid flies males might use wings primarily as visual stimuli (Puniamoorthy et al., 2009). In many species of sepsid flies, rapid wing-waving can be observed during the mating process (Puniamoorthy, 2014). Functional differences during courtship may thus explain differing selection regimes on aspects of wing shape between both clades.

Trait-size and condition-dependency of femur and wing morphology

The shape of the male femur strongly correlated with trait centroid size within all the study lineages. In particular PC1 showed a strong and positive allometric relationship and strong directional sexual selection. Bonduriansky highlighted, that male sexual traits matching these two conditions, often are also highly condition-dependent due to a resource-allocation trade-off (Bonduriansky & Day, 2003; Bonduriansky, 2007). In the closely related *S. punctum*, linear measurements of femur width and length revealed a strong positive shape allometry, but detected no significant condition-dependency (Dmitriew & Blanckenhorn, 2014). Nevertheless, the common garden experiment presented in this study revealed a significant effect of temperature on male femur size. Flies which developed under the low temperature regime developed bigger femurs, following the Temperature-Size-Rule, which applies for almost all ectotherms (C. Ray, 1960;

Atkinson, 1994). Repeating Dmitriews (2014) experiment, but with the two species used herein and applying a landmark based morphometric analysis method could provide interesting further insights. No temperature effect on wing size or shape was found, suggesting that femur morphology is more plastic, in its reaction to temperature, than the phenotypically more canalized wing morphology. Low temperature-dependent plasticity of the wing size and shape has also been reported for *Drosophila mercatorum*, in which maternal effects account for a much higher amount of variance than temperature effects (Kjærsgaard et al., 2007; also see: Weber, 1990).

Interestingly, for the femur, the regression slope of PC1 on centroids size was steepest in the American *S. neocynipsea* lineage, which in combination with the significant morphological differentiation among populations might reflect different selection intensities acting on trait size and the allometric component of trait shape. Furthermore, the American lineage is the only one showing selection on PC1 and size of the femur. Combining these findings, it can be hypothesized, that an amplifying effect might arise from trait linkage and directional selection on both traits, leading to faster divergence. Although the study at hand is not sufficient to fully disentangle the effects of selection acting on femur size and shape, the mating experiments with European flies indicate that shape can underly selection independent of size. Additional experiments and analyses are needed to tease apart effects of selection on different morphological aspects.

Influence of other evolutionary forces

Geographic patterns of morphological differentiation found in this study indicate a significant cline in femur size but not wing size across American populations of *S. neocynipsea*. Populations from high latitudes evolved larger femurs compared to populations originating from low latitudes. Taking into account the homogeneous sexual selection intensity found for the American lineage in the laboratory experiment, ecological adaption along an environmental gradient seems to play an additional role in the evolutionary diversification of male foreleg morphology. These findings suggest a rich interplay between natural and sexual selection acting on male secondary morphological traits. Furthermore, the outcome of this interplay can vary even between closely related species since the geographic differentiation in femur morphology of *S. cynipsea* was independent of the latitudinal origin of the populations.

No significant geographic differentiation within lineages was evident for wing shape, in spite the fact that populations harbored significant standing genetic variation, indicating that population wing shape can diverge if exposed to natural selection. Also, morphometric analysis indicated a strong phylogenetic signal in wing morphology, such that *S. cynipsea* was clearly differentiated from its

sister species *S. neocynipsea*, whereas the degree of differentiation between American and European *S. neocynipsea* was much smaller. This implies, that wing shape may evolve largely neutral due to mutation, drift and gene flow. Similar conclusions have been obtained from clinal studies of *Drosophila* species (Gilchrist et al., 2000; Gilchrist & Partridge, 2001). If wing shape in Sepsid flies indeed evolves neutrally as inferred from the phylogeographic analysis, the significant selection differentials for wing shape, revealed by the mating experiments, require explanation. One possibility is that sexual selection on wing shape is much weaker than selection acting on male foreleg morphology and thus gene flow may override signatures of sexual selection in wing but not femur morphology. However, in light of the use of highly inbred lines in the mating experiment, it is also possible, that selection on genetically correlated traits might explain the significant selection differentials to some extent. Although the lines were outbred for one generation to increase genetic variation, linkage disequilibrium should still be much larger compared to the situation in the field. Future studies investigating sexual selection within natural populations of *S. cynipsea* and *S. neocynipsea* are highly promising to solve this issue.

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Appendix

species	continent	population	latitude	longitude	temperature	lines	individuals
S. neocynipsea	America	Yellowstone (Wyoming)	44.60	-110.50	18	8	20
					24	8	20
		Charlottetown (PEI)	46.23	-63.13	18	4	20
					24	4	20
		Lexington (Kentucky)	38.04	-84.50	18	10	20
					24	11	20
		Zephyr cove (Nevada)	39.00	-119.57	18	6	20
					24	8	20
		Syracuse (New York)	42.94	-76.90	18	7	20
					24	5	20
		Belgrade (Montana)	45.47	-111.11	18	4	10
					24	4	10
		Tucson (Arizona)	32.13	-110.55	18	5	9
					24	9	18
		Sheridan (Wyoming)	44.48	-106.58	18	3	9
Total						96	256
	Europe	Sörenberg (Switzerland)	46.87	8.27	18	10	20
					24	10	20
		Ticino (Switzerland)	46.25	8.70	18	6	12
					24	6	12
		Wallis (Switzerland)	46.53	8.35	18	5	18
					24	7	20
Total						44	102
S.cynipsea		Sörenberg (Switzerland)	46.87	8.27	18	10	20
					24	10	20
		Ticino (Switzerland)	46.25	8.70	18	5	11
					24	9	16
		Zürich (Switzerland)	47.22	8.32	18	3	20
					24	3	12
		Killin (N UK)	56.11	-3.90	18	7	20
					24	6	20
		Reading (S UK)	51.27	-0.58	18	5	20
					24	4	20
		Pehka (Estonia)	59.48	26.37	18	6	14
					24	7	12
		Petroia (Italy)	43.21	12.34	18	6	14
					24	7	14
Total						88	233
Grand Total						288	591

Appendix table 1: Sampling locations and sample size of the populations used in the study to asses geographic patterns of quantitative genetic differentiation.

species	continent	population	repetitions	mated	unmated	total individuals
Neocynipsea	America	Lexington (Kentucky)	4	12	25	37
		Ramona (California)	3	10	18	28
		Yellowstone NP (Wyoming)	3	13	15	28
		Syracuse (New York)	4	14	26	40
	Europe	Sörenberg (Switzerland)	4	9	25	34
		Ticino (Switzerland)	2	4	15	19
		Wallis (Switzerland)	4	6	35	41
		Zürich (Switzerland)	3	6	23	29
Cynipsea		Borgonovo(Italy)	4	16	23	39
		Ludwigshafen(Germany)	3	3	24	27
		Petroia(Italy)	3	5	23	28
		Zürich (Switzerland)	3	11	15	26
total			40	109	267	376

Appendix table 2: Number of matings and individuals for the populations used in the laboratory mating experiment to asses sexual selection.

Statement of Authorship

I declare that I have used no other sources and aids other than those indicated. All passages quoted from publications or paraphrased from these sources are indicated as such, i.e. cited and/or attributed. This thesis was not submitted in any form for another degree or diploma at any university or other institution of tertiary education.

Zurich, 30. October 2016

Julian Baur